HIS PAGE BLANK (USPTO)

09/982940
#5

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/00 — A1

(11) International Publication Number: WO 90/12095

(43) International Publication Date: 18 October 1990 (18.10.90)

(21) International Application Number: PCT/US90/01722

(22) International Filing Date: 2 April 1990 (02.04.90)

(30) Priority data:
331,816 — 3 April 1989 (03.04.89) — US

(60) Parent Application or Grant
(63) Related by Continuation
US — 331,816 (CON)
Filed on — 3 April 1989 (03.04.89)

(71) Applicant *(for all designated States except US)*: PURDUE RESEARCH FOUNDATION [US/US]; Division of Sponsored Programs, 328 ENAD, West Lafayette, IN 47907 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : LOW, Philip, Stewart [US/US]; 128 Knox Drive, West Lafayette, IN 47906 (US). HORN, Mark, Alan [US/US]; 1126-0 Hawkins Graduate House, West Lafayette, IN 47907 (US). HEINSTEIN, Peter, Frederick [US/US]; 1013 East 800, West Lafayette, IN 47906 (US).

(74) Agents: CONARD, Richard, D. et al.; Barnes & Thornburg, 11 South Meridian Street, Suite 1313, Indianapolis, IN 46204 (US).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH + (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: METHOD FOR ENHANCING TRANSMEMBRANE TRANSPORT OF EXOGENOUS MOLECULES

(57) Abstract

A method is provided for enhancing transmembrane transport of exogenous molecules. A complex between a water soluble vitamin and an exogenous molecule is formed and contacted with the cell membrane thereby initiating receptor mediated transmembrane transport of the vitamin complex. The transmembrane transport of exogenous molecules including proteins and polynucleotides, as vitamin complexes, has been promoted in plant, mammalian, and bacterial cells.

+ See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic			SN	Senegal
CG	Congo	KR	Republic of Korea	SU	Soviet Union
CH	Switzerland	LI	Liechtenstein	TD	Chad
CM	Cameroon	LK	Sri Lanka	TG	Togo
DE	Germany, Federal Republic of	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

METHOD FOR ENHANCING TRANSMEMBRANE TRANSPORT OF EXOGENOUS MOLECULES

FIELD OF INVENTION

This invention relates to a method for enhancing transmembrane transport of exogenous molecules. The method takes advantage of (1) the multiplicity of location and receptors in the membrane surfaces of most cells and (2) the associated receptor mediated transmembrane processes. A complex between a water soluble vitamin and an exogenous molecule is formed and contacted with the membrane surface thereby initiating receptor mediated transmembrane transport of the vitamin complex. The transmembrane transport of exogenous molecules including proteins and polynucleotides, as vitamin complexes, has been promoted in plant, mammalian, and bacterial cells.

BACKGROUND AND SUMMARY OF THE INVENTION

Transmembrane transport of exogenous or nutrient molecules is critical for normal cell function. Because practitioners have recognized the importance of that fundamental cellular process to many areas of medical and biological science, including drug therapy and DNA transfection, there has been significant research efforts directed to the understanding and application of such processes. Transmembrane delivery of specific molecules has been encouraged through the use of protein carriers, antibody carriers, liposomal delivery systems, electroporation, direct injection,

cell fusion, viral carriers, osmotic shock, and calcium-phospate mediated transfection. However, many of those techniques are limited in both the types of cells and the conditions of use for successful transmembrane transport of exogenous molecular species. Further, many of these known techniques are limited in the type and size of exogenous molecule that can be transported across a membrane without loss of bioactivity.

One method for transmembrane delivery of exogenous molecules having a wide applicability is based on the mechanism of receptor mediated endocytotic activity. Unlike many other methods, receptor mediated endocytotic activity can be used successfully both in vivo and in vitro. Receptor mediated endocytosis involves the movement of ligands bound to membrane receptors into the interior of an area bounded by the membrane through invagination of the membrane. The process is initiated or activated by the binding of a receptor specific ligand to the receptor. Many receptor mediated endocytotic systems have been characterized, including galactose, mannose, mannose 6-phosphate, transferrin, asialoglycoprotein, transcobolamin (vitamin B-12), α-2 macroglobulins, insulin, and other peptide growth factors such as epidermal growth factor (EGF).

Receptor mediated endocytotic activity has been utilized for transmembrane delivery of exogenous molecules such as proteins and nucleic acids. Generally, the ligand is chemically conjugated by covalent, ionic or hydrogen bonding to exogenous

molecule of interest, (i.e., the exogenous compound) forming a conjugate molecule having a moiety (the ligand portion) that is still recognized in the conjugate by a target receptor. Using this technique the phototoxic protein psoralen has been conjugated to insulin and internalized by the insulin receptor endocytotic pathway (Gasparro, Biochem. Biophys. Res. Comm. 141(2), pp. 502-509, Dec. 15, 1986); the hepatocytes specific receptor for galactose terminal asialo-glycoproteins has been utilized for the hepatocytes-specific transmembrane delivery of asialoorosomucoid-poly-L-lysine non-covalently complexed to a DNA plasmid (Wu, G.Y., J. Biol. Chem., 262(10), pp. 4429-4432, 1987); and the cell receptor for epidermal growth factor has been utilized to deliver polynucleotides covalently linked to EGF to the cell interior (Myers, European Patent Application 86810614.7, Filed December 29, 1986, Publication Date June 6, 88).

The method of the present invention enhances the transmembrane transport of an exogenous molecule across a membrane having receptors for water soluble vitamins that initiate transmembrane transport following binding with a water soluble vitamin or a pharmacologic agent that mimics the binding of a water soluble vitamin. The present method has been successfully applied to mammalian, plant, and bacterial cells. The vitamin receptor mediated transmembrane transport forming the basis of this invention is initiated by the binding of water soluble vitamins, such as biotin, ascorbic acid, cobalamine, or folates, to their

respective receptor associated with the membrane. A preferred target receptor for the method of the present invention is the biotin receptor. Biotin is a necessary cellular growth factor that has been found to be preferentially bound by biotin receptor proteins associated with cellular membranes. Biotinylating reagents suitable for covalently bonding a biotin moiety to polynucleotides, proteins, or other desired molecules are commercially available. The binding of the vitamin moiety to its cell surface receptor initiates transmembrane transport of the water soluble vitamin or, in the case of the present invention, the complex consisting of the vitamin and the exogenous molecule.

The present invention makes use of a receptor-mediated transmembrane transport to deliver exogenous molecules complexed with the water soluble vitamin, across a membrane. A complex is first formed between a water soluble vitamin and a predetermined exogenous molecule. The complex is then contacted with a cell having receptors for water soluble vitamins and an associated receptor mediated transmembrane transport activity for a time sufficient to permit transmembrane transport of the complex by water soluble vitamin receptor mediated transmembrane transport activity. In this manner, exogenous molecules are either transported, or transported at an enhanced rate, across the membrane.

The method of the present invention is particularly useful in increasing the internalization yields (cellular uptake) of exogenous molecules that normally are resistant to cellular internalization.

Proteins and polynucleotides previously recognized as difficult to move across cell membranes can be internalized by the method of the present invention. For example, transfection and expression of an encoded protein product by an internalized biotin-complexed functional gene has been demonstrated. Biotin, conjugated with a DNA plasmid containing a gene sequence coding for chloramphenicol acetyltransferase (CAT), was transported into E. coli via a biotin receptor mediated endocytotic pathway and expressed. Transport of biotinylated protein products into both mammalian and plant cells has also been achieved in both in vivo and in vitro systems.

The method of the present invention can also be accomplished utilizing chemical analogues or derivatives of water soluble vitamins that are cross reactive with a water-soluble-vitamin receptor.

DETAILED DESCRIPTION OF THE INVENTION

The method of the present invention requires the presence of appropriate receptors for water soluble vitamins associated with a membrane. The membrane can either define an intracellular volume such as the endoplasmic reticulum or other organelles such as mitochondria, or alternatively can define the boundary of the cell. Transmembrane transport across a cell boundary commonly occurs by an endocytotic transport mechanism. Generally, it has been found that water soluble vitamin receptors mediate cellular internalization of water soluble vitamins through

endocytotic activity. The receptors can be natural constituents of the cell or they can be emplaced in the cell membrane by external physical manipulation. Alternatively, expression of an inserted foreign gene for the protein or apoprotein corresponding to the water soluble vitamin receptor by a transfected cell can ensure the presence of a water soluble vitamin receptor on a target cell.

Water soluble vitamins known or believed to have suitable cellular receptors for purposes of the present invention include but are not limited to biotin, biotin analogues such as 6-N-biotinyl-L-lysine (biocytin), biotin sulfoxide, oxybiotin (oxobiotin), 5,6,-dimethylbenzimidazoloylcyanocobamide (cyanocobalamin - vitamin B-12), 5,6,-dimethylbenzimidazoloylaquaocobamide (aquocobalamin - vitamin $B\text{-}12_a$), 5,6,-dimethylbenzimidazoloylhydroxocobamide (hydroxocobalamin - vitamin $B\text{-}12_b$), adenosylcobalamin, methylcobalamin, folic acids such as folacin, methotrexate, pteropolyglutamic acid, pteridines, niacin, pantothenic acid, riboflavin, and thiamin.

Preliminary experiments using the water soluble vitamin pyridoxine showed little uptake potentiating activity. It is possible that pyridoxine and pyridoxine analogues are not suitable for use in accordance with the present invention.

Because of the ready availability of biotinylating reagents and biotinylating methods suitable for use with peptides, proteins,

oligonucleotides, and polynucleotides, a preferred water soluble vitamin for the purposes of the present invention is biotin. Biotin is also a preferred water soluble vitamin because it is a necessary growth factor for a wide variety of cells, and biotin receptors that mediate endocytotic activity have been identified in mammalian, plant, and bacterial cells.

Formation of a complex between a water soluble vitamin such as biotin and an exogenous molecule of interest is readily accomplished for a great many molecules and macromolecules. Biotin moieties can be easily conjugated to proteins by making the carboxyl group of biotin reactive toward the free amino groups of the proteins. A biotinylating reagent such as D-biotin-N-hydroxy-succinimide ester or biotinyl-p-nitrophenyl ester can be used. The activated ester reacts under mild conditions with amino groups to incorporate a biotin residue into the desired molecule. The procedure to be followed for biotinylating macromolecules using D-biotin-N-hydroxy-succinimide ester is well known in the art (Hofmann et al., J.Am.Chem.Soc. 100, 3585-3590 (1978)). Procedures suitable for biotinylating an exogenous molecule using biotinyl-p-nitrophenyl ester as a biotinylating reagent are also well known in the art (Bodanszk et al., J.Am.Chem.Soc. 99, 235 (1977)). Other reagents such as D-biotinyl-ε-aminocaproic acid N-hydroxy-succinimide ester in which ε-aminocaproic acid serves as a spacer link to reduce steric hindrance can also be used for the purposes of the present invention.

Oligonucleotides and polynucleotides can also be biotinylated using both indirect and direct methods. Indirect methods include end-labeling of a polynucleotide with a biotinylated nucleotide, or nick translation that incorporates biotinylated nucleotides. Nick translation or end labeling of DNA can be accomplished using methods described in Maniatis et al., Molecular Cloning: A Laboratory Manual, pp. 109-116, Cold Spring Harbor Press (1982).

Direct methods refer to those procedures in which biotin is directly attached to a target polynucleotide using a biotinylating reagent. Photoactivatible reagents such as the acetate salt of N-(4-azido-2-nitrophenyl)-N-(3-biotinylaminopropyl)-N-methyl-1,3-propanediamine (photobiotin) can be used to biotinylate DNA according to the method of Forster et al., Nuc. Acids Res. 13:745-761. An alternative method uses a biotin hydrazide reagent in a bisulfite catalyzed reaction capable of transamination of nucleotide bases such as cytidine according to the method described by Reisfeld et al., B.B.R.C. 142:519-526 (1988). This method simply requires a 24 hour incubation of DNA or RNA with biotin hydrazide at 10mg/ml in an acetate buffer, pH 4.5, containing 1 M bisulfite. Biotin hydrazide can also be used to biotinylate carbohydrates containing a free aldehyde.

Water soluble vitamin receptor mediated cellular uptake of biotinylated polynucleotides provides an alternative mechanism for transfection of cells. The technique of the present invention is particularly

valuable because it is applicable to certain cell types, such as plant cells, which are normally resistant to standard transfection techniques. Delivery of foreign genes to the cell interior can be enabled or enhanced by the present invention. Once delivered to the cellular interior, these foreign genes can be inserted and expressed with the aid of a natural or exogenous promoter to produce a desired protein. In addition to proteins, other useful macromolecules can be produced. For example, an antisense-RNA sequence capable of binding interference with endogeneous messenger RNA.

The delivery of proteins and other non-nucleotide molecules by water soluble vitamin receptor mediated uptake is also useful. Antibodies, bioactive peptides, toxic peptides, or pharmaceutically valuable peptides can be delivered to the cellular interior by means of the present invention. This is of particular value for in vivo, therapeutic applications involving the delivery of molecules that are not normally internalized by a target cell.

The following examples are provided to illustrate further the range of exogenous molecules and cell types to which the method of the present invention can be applied.

Example 1 - RAT PHEOCHROMOCYTOMA CELL UPTAKE OF BIOTIN CONJUGATED INSULIN:

Rat pheochromocytoma (PC-12) cells were obtained from America Type Culture Collection and were grown (37°C, 5% CO_2 in humidified air) attached to plastic flasks for 2 to 3 weeks until confluent in a

medium of 85% RMPI 1640, 10% v/v heat inactivated horse serum, and 5% fetal calf serum containing 1% streptomycin-penicillin.

Biotin and fluorescein labeled insulin was prepared. To 1 ml of a 1 mg/ml solution of insulin protein in phosphate buffered saline was added simultaneously 100 µl of a 1 mg/ml solution of fluoroscein isothiocyanate (FITC) in dimethylformamide (DMF) and 100 µl of a 1 mg/ml solution of N-hydroxysuccinimido biotin in dimethylsulfoxide (DMSO). The two labeling reagents were allowed to react at room temperature for 4 hours, after which the unreacted reagents were quenched with 10 µl ethanolamine. The quenched reaction mixture was then dialyzed against double distilled water until unreacted fluorescein derivatives no longer dialyzed into the water. The covalent attachment of biotin and fluorescein to the desired protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis.

As a control, non-biotinylated fluorescein labeled insulin was prepared. 1 ml of a 1 mg/ml solution of insulin was added 0.5 ml of a 1 mg/ml solution of fluorescein isothiocyanate (FITC) in dimethylformamide (DMF). The reaction was allowed to proceed for 4 hours in the dark at room temperature. After 4 hours the reaction was quenched with 10 µl ethanolamine, and the labeled insulin solution was dialyzed against double distilled water until unreacted FITC no longer appeared in the solution.

The rat PC12 cells were grown in modified RMPI 1640 medium as a monolayer on the bottom of a culture flask. Before removing the cells, the monolayer was washed with a 20 ml portion of fresh Locke's solution. The cells were then displaced into 20 ml of the Locke's solution by gentle agitation with a stream Locke's solution. The suspended cells were pelleted by centrifugation at 10,000 x g for 10 seconds and after resuspending in Locke's solution in separate polycarbonate tubes (40ml/tube) to a final density of 1.14×10^6 cells/ml, the following amounts of proteins were added to the cell suspensions: 40 µg fluorescein-labeled insulin was added to the first tube, and to the control tube was added 40 µg biotin-conjugated insulin labelled with fluorescein. The tubes were allowed to incubate at 37°C. At intervals of 5, 15 and 33 minutes, 0.5 ml of each cell suspension was removed and pelleted at 10,000 x g for 10 seconds. The cell pellet was washed and repelleted twice in 1 ml Locke's solution and then fixed by addition of 200 µl of a 2% formalin solution in phosphate buffered saline. Thirteen microliters of the fixed cell suspension was then added to a microscope slide and viewed with the fluorescent microscope to detect internalized proteins. No evidence of internalization was noted for the fluorescein labelled insulin acting as a control. Cellular internalization was indicated for the biotinylated insulin labelled with fluorescein, with the amount internalized increasing with time.

Example 2 - RAT PHEOCHROMOCYTOMA CELL UPTAKE OF BIOTIN CONJUGATED HEMOGLOBIN:

Following the same general procedure set forth in Example 1 hemoglobin was biotinylated, and the biotinylated form was shown to be preferentially internalized by rat pheochromocytoma cells as compared to non-biotinylated hemoglobin.

Example 3 - SOYBEAN CELL UPTAKE OF BOVINE SERUM ALBUMIN:

Soybean cell suspension cultures of Glycine max Merr Var Kent were maintained by transferring cells to fresh W-38 growth medium every 7 days.

To 20 ml of a suspension culture of soybean cells was added 10 µg of either fluorescein-labeled (control) or fluorescein and biotin labelled bovine serum albumin. The cells were allowed to incubate for up to 6 hours. At varying time intervals 1 ml of the cell suspension was filtered to remove the growth medium, washed with 50 ml fresh growth medium, and resuspended in 20 ml of the same medium. The cell suspension was then viewed with a flourescent microscope to determine whether cellular internalization of the labelled bovine serum albumin had occurred. Cellular internalization was indicated only for biotinylated bovine serum albumin.

Example 4 - SOYBEAN CELL UPTAKE OF INSULIN:

Following the same general procedure set forth in Example 3 insulin was biotinylated, and the biotinylated form of insulin was shown to be preferentially internalized by soybean cells as compared to non-biotinylated insulin.

Example 5 - SOYBEAN CELL UPTAKE OF HEMOGLOBIN:

Following the same general procedure set forth in Example 3 hemoglobin was biotinylated, and the biotinylated form of hemoglobin was shown to be preferentially internalized by soybean cells as compared to non-biotinylated hemoglobin.

Example 6 - CARROT CELL UPTAKE OF BOVINE SERUM ALBUMIN:

Carrot cells of wild type origin were established and maintained in MS growth medium supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid. Bovine serum albumin was labelled with fluorescein alone as a control or with fluorescein and biotin following the procedures detailed in Example 3. The carrot cells were then incubated in the presence of the respective labelled bovine serum albumin for 7 hours. All other conditions were the same as those described in Example 3 above. Cellular internalization was found only in those cells contacted with biotin labelled bovine serum albumin.

Example 7 - CARROT CELL UPTAKE OF INSULIN:

Following the same general procedure set forth in Example 6 insulin was biotinylated, and the biotinylated form was shown to be preferentially internalized by carrot cells as compared to non-biotinylated insulin.

Example 8 - CARROT CELL UPTAKE OF HEMOGLOBIN:

Following the same general procedure set forth in Example 6 hemoglobin was biotinylated, and the biotinylated form was shown to be preferentially internalized by carrot cells as compared to non-biotinylated hemoglobin.

Example 9 - SOYBEAN CELL DEGRADATION OF HEMOGLOBIN:

To determine whether hemoglobin was rapidly degraded following cellular internalization by transmembrane transport, soybean cells were allowed to internalize and metabolize biotinylated hemoglobin for a period of 8 hours under conditions described in Example 5, after which the soybean cells were rapidly homogenized in a sodium dodecyl sulfate solution to disaggregate and denature all protein material. The solubilized polypeptides were separated according to molecular weight by polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose paper. The positions of the biotin-labeled peptides were then visualized on the nitrocellulose blot by staining with horseradish peroxidase-linked avidin and the colored substrate, p-chloronaphthol. All of the biotin-linked material was found to migrate with an apparent molecular weight of ~16,000 daltons, about equal to the molecular weight of the parent globin chains of hemoglobin, indicating no breakdown of the parent globin chains had occurred during the 8 hour incubation period.

Example 10 - IN VIVO DELIVERY TO RATS OF SOYBEAN TRYPSIN INHIBITOR:

Soybean trypsin inhibitor (SBTI) (~6 mg) was labeled with radioactive ^{125}I using 8 iodobeads (Bio Rad) in 1 mL buffer which was then dialyzed to remove unreacted ^{125}I. After dividing into two equal fractions, one fraction was biotinylated with N-hydroxysuccinimidyl biotin and the other fraction was left as an unmodified control. Mice (~25 g) were then

injected with either the biotinylated SBTI or the control SBTI by insertion of a hypodermic syringe containing a 25 gauge needle into the tail vein of the mouse. After 15 minutes, each mouse was sacrificed and then perfused with heparin-containing isotonic saline via the direct cardiac influx and efflux method. When the various tissues appeared to be blood-free, the perfusion was terminated and each tissue/organ was removed, weighed, and counted for ^{125}I-SBTI in a gamma counter. Although some radioactivity was detected in the mice treated with non-biotinylated ^{125}I-SBTI, between 4 and 100 times more ^{125}I-SBTI was found in the mice treated with biotinylated SBTI, indicating successful in vivo delivery to murine cellular tissue.

Counts per minute/gram wet weight

Tissue	Control SBTI	Biotin SBTI
Liver	535	1967
Lung	107	2941
Kidney	5152	8697
Intestine	0	700
Muscle	0	1065
Heart	0	739
Brain	0	267

Example 11 - SOYBEAN TRANSFECTION OF SALMON SPERM DNA:

Protein free salmon-sperm DNA, either in a highly polymerized form ($\geq$ 50,000 base pair length) or

in a sheared form (≤ 500 base pair length), was transaminated at the cytosine residues. The transaminated DNA (1 mg) was labeled with fluorescein via the addition of 0.5 mg of fluorescein isothiocyanate (FITC) in dimethylsulfoxide (DMSO). The resulting reaction mixture was divided into two portions and the labeling reaction was quenched in one portion by addition of 10 µL of ethanolamine. This quenched portion served as the non-biotinylated control. The remaining DNA was then covalently labeled with biotin via reaction with 0.5 mg of N-hydroxysuccinimidyl biotin in DMSO. After purification, the two derivatives (1 µg/ml) were separately incubated with soybean suspension culture cells at room temperature for 6 hours and then the cells were washed with 50 ml fresh growth medium and observed by fluorescence microscopy. Only the biotinylated DNA entered the soybean cells.

Example 12 - E. COLI TRANSFECTION AND EXPRESSION OF AMPICILLIN RESISTANT GENE:

Plasmid DNA (pUC8) was biotinylated via nick translation in the presence of biotin-14-dATP using a commercially available nick translation kit (Bethesda Research Laboratories). The biotinylated DNA and unmodified DNA (1 µg) were added to E. coli strain Cu 1230 that had been made competent by treatment with $MgCl_2$ and $CaCl_2$ following the method of Maniatis et al., Molecular Cloning: A Laboratory Manual, pp. 250-251, Cold Spring Harbor Press (1987). After transformation, the successful transformants were selected by plating cells on LB media which contained 50

µg/ml ampicillin and then incubated overnight at 37°C. Colonies which survived the ampicillin were counted and the transformation efficiency was determined. The number of surviving E. coli colonies was at least 100-fold greater in E. Coli transformed with the biotinylated plasmids.

WHAT IS CLAIMED IS:

1. A method for enhancing transport of an exogenous molecule into a plant cell, said method comprising the step of contacting the plant cell with the exogenous molecule complexed with a water soluble vitamin or water soluble vitamin receptor binding agent for a time sufficient to permit transmembrane transport of said complex.

2. The method of claim 1 wherein the water soluble vitamin is biotin or analogs thereof.

3. The method of claim 1 or 2 wherein the exogenous molecule is a nucleic acid.

4. The method of claim 1 or 2 wherein the exogenous molecule is a protein.

5. A method for enhancing transport of a nucleic acid into a cell, said method comprising the step of contacting the cell with the nucleic acid complexed with a water soluble vitamin or water soluble vitamin receptor binding agent for a time sufficient to permit transmembrane transport of said complex.

6. The method of claim 5 wherein the water soluble vitamin is biotin or analogs thereof.

7. The method of claim 5 or 6 wherein the cell is an animal cell.

8. The method of claim 5 or 6 wherein the cell is a plant cell.

9. The method of claim 5 or 6 wherein the cell is a prokaryote.

10. A method for enhancing transport of an exogenous molecule into cellular tissue of a host having

a circulatory system, said method comprising the step of injecting the host with the exogenous molecule complexed with a water soluble vitamin or water soluble vitamin receptor binding agent.

11. The method of claim 10 wherein the water soluble vitamin is biotin or analogs thereof.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01722

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00
U.S. CL.: 435/6,7

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S. CL.	435/6,7, 172.3, 536/27 935/56, 58, 52

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

APS: biotin, introduc., cell(s)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,683,205 issued 28 July 1987, (KATSUMATA, ET AL.), see entire document.	1-11
Y	Methods of Biochemical Analysis, volume 26 issued 1978, (BAYER ET AL.), "The use of the avidin-biotin complex as a tool in molecular biology", see pages 1-45.	1-11
Y	Biochemics et Biophysic Acts Volume 721 issued 1982 (CHALIFOUR ET AL.), "The characterization of the uptake of avidin-biotin complex by Hela Cells", see pages 64-69.	1-11
Y	Annual Review of Physiology, Volume 43 issued 1981, (PASTAN ET AL.), "Receptor-mediated endocytosis of hormones in cultured cells", See pages 239-250.	1-11

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
22 JUNE 1990	01 AUG 1990

International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	MICHELLE S. MARKS

Form PCT/ISA/210 (second sheet) (May 1986)